THE RNA-DEPENDENT RNA POLYMERASE OF COWPEA

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1. Introduction

Consensus has now been reached for several plants that they contain RNA-dependent RNA polymerases of similar properties to the RNA replicases produced in those plants upon virus infection [1–8]. The main difference is that the polymerases in healthy plants are reported as largely soluble and RNA-dependent, while 'viral RNA replicases' are largely membrane-bound and associated with the endogenous virus RNA template and products. We have recently adduced strong evidence that the RNA polymerase from uninfected and tobacco necrosis virus (TNV)-infected tobacco plants is identical with the 'tobacco mosaic virus RNA replicase' and probably also with the 'alfalfa mosaic virus RNA replicase' produced in these plants upon infection with the respective viruses [6,9]; or in other words that the tobacco enzyme serves as replicase for any infecting virus, and becomes or remains more membrane-associated when acting in this capacity. This concept is not yet fully accepted even by those who agree that the properties of the free enzyme from healthy and virus-infected plants are not distinguishable [6,8,9]. We have now designed an experimental approach in further test of this hypothesis by studying the RNA polymerase of a different host for TNV, namely cowpea, again comparing the properties of the enzyme from healthy and infected plants, as well as comparing those of the cowpea with those of the tobacco enzyme. If the enzyme is host-specific we would expect detectable differences between preparations from tobacco and cowpea, even if both are infected by the same virus, and we would expect no differences in the free enzyme from healthy and infected cowpea plants. Our results fully confirm our belief that different plants carry different RNA-dependent RNA polymerases which serve without presently detectable changes in the replication of different viral RNAs.

The isolation from cowpeas of what was termed cowpea mosaic virus RNA polymerase has been reported [10], and very recently there appeared a study of cowpea chlorotic mottle virus RNA replicase also isolated from cowpeas [11]. The properties of the enzyme isolated after 3 different virus infections, as well as from uninfected cowpeas, in 3 different laboratories are in general accord, further confirmation that the enzyme is host-specific without any indication of a virus-induced modification of its properties.

2. Materials and methods

Except for the use of cowpeas (*Vigna unguiculata*) the methods and materials are all identical to those used in [3,4] dealing with the tobacco enzyme and will not be detailed here. At 8–10 days after planting, the primary leaves of cowpea were inoculated with crude sap from plants infected with TNV, and the plants placed in a controlled-environment chamber at 25°C with 20 h daily illumination with fluorescent light. At 3 days post-inoculation, directly inoculated leaves with necrotic local lesions were used as a source of enzyme. Uninfected controls consisted of comparable leaves which were mock-inoculated with 0.1 M phosphate (pH 7.0) buffer. The plants were frozen at −70°C, then ground in 50 mM Tris–HCl (pH 8.5), 100 mM NH_4Cl, 90 mM 2-mercaptoethanol, 2 mM EDTA, 5% glycerol. The miracloth filtrate was

mixed and stirred with an equal volume of saturated $(NH_4)_2SO_4$, the residue again extracted with 50% saturated $(NH_4)_2SO_4$, and that residue extracted 2 or 3 times with the same buffer lacking $(NH_4)_2SO_4$. This 'low-salt extract' is subjected to polyethylene-glycol–dextran 2-phase separation (PEG-D enzyme), followed by sucrose gradient centrifugation, DEAE–Sephadex fractionation and isoelectric focusing.

The enzyme activity is tested by the usual method with 3 unlabeled nucleoside triphosphates and [^{3}H]-UTP and studying the macromolecular ^{3}H incorporation.

3. Results

3.1. *Differences between cowpea and tobacco enzymes*

The low-salt extract of healthy or infected cowpeas contained only ~70% of the total enzyme activity, compared to 97% for the tobacco enzyme. Apparently the high-salt treatment is less effective in dissociating the cowpea enzyme from the template- and membrane-bound state, than it is in the case of tobacco. The yield after the PEG-D step was also much lower for cowpea than tobacco (9% versus 43%), suggesting a more labile cowpea enzyme. The activity of both enzymes was greatly increased by, but not dependent upon, the addition of RNA at this stage of purification (table 1).

When the effect of various Mg^{2+} and Mn^{2+} concentrations on the activity of the enzyme from the 2 sources was studied, a clearcut difference was noted in the sharp optimum of [Mg^{2+}] in tobacco (see fig.1); the diminishing activity at higher concentrations which is not shown by the cowpea enzyme was not due to the [Cl^-], since chloride was found to depress both enzyme activities only at much higher concentrations (>0.2 M). Mn^{2+} was with both enzymes considerably less active, and optimal at lower concentration than Mg^{2+}, but also showed a different concentration dependence; use of both Mg^{2+} and Mn^{2+} caused diminished incorporation. Very similar results with Mg^{2+} and Mn^{2+} were obtained for cowpea enzyme [10] and for the tobacco enzyme [8,9].

Another marked difference was detected when comparing the template preference of the 2 enzymes after further purification by DEAE–Sephadex chromatography. While all plant RNA-dependent RNA polymerases act on all RNA templates, definite quantitative differences are regularly noted. Thus the tobacco enzyme is stimulated more by turnip yellow mosaic virus (TYMV) RNA than by other viral RNAs [1–3]. In contrast, for the cowpea enzyme tobacco mosaic virus (TMV) RNA is the most active of the RNAs tested (table 2); this was also observed [12]. It should be remembered that TNV is the virus used, if any, in increasing the production of the enzyme.

In all these regards, as well as those discussed in

Table 1
RNA polymerase activities on crude fractions obtained from TNV-infected and uninfected leaves

Plants	Assay RNA[b]	[^{3}H]UMP incorporation (cpm/g leaf)[a]			
		Low salt extraction		PEG-D enzyme	Recovery of PEG-D enzyme (%)
		Extract	Residue		
Infected tobacco	–	102 320	4240	3320	2
	+	153 720	4840	83 580	54
Uninfected tobacco	–	16 573	–	4340	26
	+	28 325	–	9174	32
Infected cowpea	–	56 610	22 866	1360	2
	+	72 000	30 900	7260	10
Uninfected cowpea	–	48 421	21 767	1349	3
	+	56 363	31 547	4670	8

[a] 153 720 cpm corresponds to 0.3 nmol
[b] TYMV RNA, 10 μg

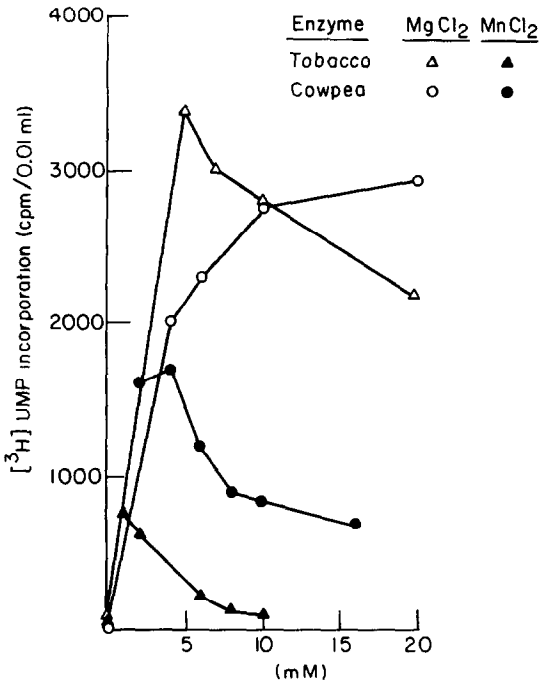

Fig.1. Effect of $[Mg^{2+}]$ and $[Mn^{2+}]$ on activity of RNA polymerase from tobacco and cowpea tested in presence of 10 μg TYMV RNA. This experiment was done with enzyme isolated by two-phase separation (PEG-D). Similar results were obtained after DEAE–Sephadex chromatography (see footnote c, table 2).

the following section, the enzyme from infected and uninfected cowpeas acted alike.

3.2. *Presently indistinguishable properties of cowpea and tobacco enzyme*

Upon sucrose gradient centrifugation both enzymes sediment alike, indicating similar molecular weights (175 000 ± 15 000). Preliminary isoelectric focusing experiments indicate the same sharp peak of the activity; due to the unfavorably steep pH gradient under the conditions used. This can only be regarded as an approximation, both enzymes showing isoelectric points at pH 6 ± 1. This similarity is borne out by their eluting from DEAE–Sephadex at the

Table 2
Effect of various RNAs on [^{3}H]UMP incorporation of RNA polymerases[a] from TNV-infected and uninfected tobacco and cowpea plants

	[^{3}H]UMP incorporation (cpm)		
RNA (10 μg)	TNV-infected cowpea	Uninfected cowpea[b]	Uninfected tobacco[c]
None	133	112	94
Brome mosaic virus	2355	2391	739
Phage Qβ	2420	2233	691
Tobacco mosaic virus	3132	3132	610
Tobacco necrosis virus	1937	1909	558
Turnip yellow mosaic virus	1602	1248	968
Yeast	228	138	593

[a] Purified after high-salt treatment by PEG-D followed by DEAE–Sephadex chromatography

[b] Normalized to same activity as enzyme from infected cowpea

[c] This represents an older enzyme preparation that has lost activity upon storage; the relative superiority of TYMV RNA as template, compared to other RNAs has been reported for both TNV-infected and uninfected tobacco [1,3]. The specific activity of freshly purified tobacco (healthy and infected) RNA-dependent RNA polymerase at this stage of purification was reported to be about 1.3 nmol/mg [4]. With cowpea enzyme such analyses were not done for reasons of low enzyme recovery (see table 1); they were not regarded as essential, since only comparative data on the template activities of various RNAs were the object of the experiment

same salt molarity, 0.13 ± 0.01, somewhat lower than the concentration needed to elute the 'soluble enzyme' that still retained some template [3]. Both enzymes are precipitated between 0.3 and 0.4 saturation with ammonium sulfate; also in various other regards, such as inhibition of activity by high Cl^- but not acetate concentration, lack of activity of other divalent metals (Ca^{2+}, Cu^{2+}), inhibition by over 0.07 M K^+, lack of inhibition by actinomycin D, the enzymes resemble one another.

4. Discussion

We have shown earlier that the 'bound' and 'soluble' RNA-dependent RNA polymerase of tobacco appears to be the same enzyme, differing only in its association with template and, possibly as a consequence, in its cellular location, and the nature of its products (large and in part single-stranded for 'bound' and largely small and double-stranded for 'soluble' enzyme). We thus now utilize methods aimed at isolation of the entire enzyme activity in the soluble template-free state. This has enabled us to compare the properties of the RNA-dependent enzymes from healthy and variously virus-infected tobacco and to find them indistinguishable by all tests that were applied. We have now extended these studies to a different plant, yet infectable by one of the viruses used with tobacco (cowpea, TNV), and have arrived at the same conclusion that these enzymes are plant-specific, showing differences from one plant to another, but no differences whether virus-infected or not. The possibility of a virus-coded component becoming associated with the plant enzyme is difficult to rule out, but the unchanged sedimentation rate and lack of template specificity represent evidence against such a hypothesis. We have dealt with the recurring question how one can exclude the possibility of cryptic virus infections as accounting for the presence of RNA polymerases. One would now have to postulate 2 different cryptic viruses infecting, respectively, tobacco and cowpea, in 3–5 different locations around the world. The more searching question, what the role of this enzyme may be in the physiology of the plant, remains to be answered. We tend to hypothesize that its origin and its normal role is in the chloroplasts.

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